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Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds

Isam Ali Mohamed Ahmed [a], Isao Morishima [a], Elfadil E. Babiker [b,c,*], Nobuhiro Mori [a]

[a] United Graduate School of Agricultural Science, Tottori University, Koyama, Tottori 680-8553, Japan
[b] Tottori University, Faculty of Agriculture, Department of Microbial Biotechnology, Koyama, Tottori 680-8553, Japan
[c] University of Khartoum, Faculty of Agriculture, Department of Food Science and Technology, Khartoum North 13314, Shambat, Sudan

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ABSTRACT

The seeds of *Solanum dubium* were blended and extracted using different types of buffers. The most reliable, quick, and efficient buffer was found to be 5% NaCl in acetate buffer (pH 5.0) which was used throughout the study. The extract was filtered and fractionated twice with ammonium sulphate. The partially purified enzyme was characterised by SDS–PAGE which showed a band of molecular weight of 66 kDa with the presence of other bands of low density. When compared with other plant enzymes, *S. dubium* enzyme was found to have higher clotting and proteolytic activities. The activity of the enzyme was steadily increased with enzyme and substrate concentration. The enzyme was found to be very stable against a wide range of pH values as well as a wide range of temperature (20–90 °C). The results of substrate specificity of the enzyme showed that the partially purified enzyme preferred both hydrophilic and hydrophobic amino acid residues at P1 position. The catalytic efficiency of the purified enzyme was enhanced by an aliphatic amino acids (Leu) compared to aromatic residue (Phe) at P1 position at the same site.

1. Introduction

Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been widely used as a milk-clotting enzyme. Increasing world cheese production and consumption together with the increase of calf rennet's price and a reduced supply of calf rennet, has led to a systematic investigation for new rennet substitutes. Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactory replace calf rennet in cheese manufacture. Moreover, consumer constraints on the use of rennet have led to a growing interest in vegetable coagulants. More recently, the incidence of bovine spongiform encephalopathy (BSE) has reduced both supply and demand for bovine rennet (Roseiro, Barbosa, Ames, & Wilbey, 2003). Microbial rennet produced by genetically engineered bacteria have proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants such as *Ananas comosus* (Cattaneo, Nigro, Messina, & Giangiacomo, 1994), *Carica papaya* (Cabezas, Esteban, & Marcos, 1981), *Ficus carica* (El-Shibiny, Abd El-Salam, Rifaat, & Fahmi, 1973), *Calm viscera* (Gupta & Eskin, 1977), *Cynara cardunculus* (Heimgartner et al., 1990), and *Cynara scolymus* (Sidrach, García-Canovas, Tudela, & Rodrıguez-Lopez, 2005). Unfortunately, most of the plant rennets were found unsuitable because they produced extremely bitter cheese.

An exception to this general rule is represented by the aqueous extract of *C. cardunculus* flowers containing two aspartic acid-type proteases, namely cardosins A and B (Verissimo et al., 1995), which have been used for the manufacture of sheep milk cheese in several areas of Portugal and Spain. Some plants of the family Solanaceae such as *Solanum innacum* (Suleiman, El-Imam, & Allagabo, 1988) have been tried as sources of milk-clotting enzymes. *Solanum dubium* Fresen is an indigenous plant in central, northern, and western Sudan. It is a woody herb with a solid erect stem, green[1] in colour and about 30 cm high (Fig. 1). The stem and its branches bear numerous sharp spines of 1–3 mm in length and about 1 mm in thickness near the base. The leaves are alternate, long petiole, simple, ovate, acuminate or obtuse at the apex and pale green brown in colour while the rootlets are brown, and the root about 5 mm thick and 15 cm length. The flowers are a hermaphrodite,

* Corresponding author. Address: University of Khartoum, Faculty of Agriculture, Department of Food Science and Technology, Khartoum North 13314, Shambat, Sudan. Tel.: +249 915482977; fax: +249 185318919.
E-mail address: elfadilbabikr@yahoo.com (E.E. Babiker).

[1] For interpretation of colour in Fig. 1, the reader is referred to the web version of this article.

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Fig. 1. *Solanum dubium* whole plant (left), fruits (yellow and green) and seeds (black clusters).

actinomorphic with a yellow centre core-like structure. The fruits are grouped in clusters with exile alternately bent to bring all clusters to one side of the stem or to the branch. Its berries are globular in shape, being 1.0 cm in diameter with smooth lustrous surfaces. Unripe fruits are green and almost enclosed in spiny calyx, while the ripe fruits are yellow. The seeds are dark brown in colour, and the testa is minutely pitted.

Dairy farmers in some parts of the Sudan use the berries of *S. dubium* to make white soft cheese using goat and sheep milk. Milk coagulation takes about 2 h and the curd is pressed to remove whey. The cheese obtained has a slight bitter taste and a fragile crumbly texture. The bitterness of the cheese is probably a result of the presence of some alkaloids or unspecific proteolytic activity of the enzymes during processing due to impurities because the farmers used raw seeds. It may be possible to reduce bitterness by using a purified enzyme and by using optimum extract concentration (Yousif, McMahon, & Shammet, 1996). However, detailed and accurate studies to determine the chemical properties of the enzyme as a milk coagulant have not yet been carried out. Therefore, in this work we would like to study the properties of a partially purified enzyme from *S. dubium* seeds.

2. Materials and methods

2.1. Materials

S. dubium seeds were obtained from Shambat, Khartoum North, Sudan. The *S. dubium* fruits were collected during the season 2004/2005, sun dried and hand crushed to obtain the seeds. Standard proteins for SDS–PAGE were from Bio-Rad laboratories (Hercules, CA, USA). Synthetic peptide substrates *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide, *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and *N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide were from Sigma (Saint Louis, MO, USA), and Ac-Tyr-Val-Ala-Asp-*p*-nitroanilide was from Merck (Darmstadt, Germany). Unless otherwise stated all chemicals used in this study are of reagent grade.

2.2. Methods

2.2.1. Extraction of the enzyme

S. dubium seeds (50 g) were coarsely milled in a mortar and extracted with 500 ml using different buffers {distilled water, 5% NaCl in distilled water, 5% NaCl in sodium acetate buffer (pH 3.8 and 5.0), and 5% NaCl in tris–HCl buffer (pH 8.0)} for 1–24 h with stirring at 4 °C. The extract of each buffer was filtrated through cheesecloth and centrifuged at 12,000 rpm for 20 min. The supernatant was dialysed overnight at 4 °C against 0.1 M sodium acetate buffer (pH 5.0). Milk-clotting and protease activities as well as the protein concentration for each extract were determined.

2.2.2. Partial purification of the enzyme

2.2.2.1. Ammonium sulphate fractionation (first). The supernatant (510 ml) from the above step was brought to 35% saturation with a gradual addition of solid ammonium sulphate and allowed to stand on ice for 30 min. The resulting precipitate was removed by centrifugation at 12,000 rpm (HIMAC, type SCR 18B and CR 20B2, Hitachi Koki Co., Ltd., Tokyo, Japan) for 20 min at 4 °C and dissolved in 50 mM sodium acetate buffer (pH 5.0). The above step was repeated twice to obtain 55% and 80% saturation. The supernatant of each step was dialysed against 50 mM sodium acetate buffer (pH 5.0) for 24 h with frequent changes of the buffer. After dialysis, the solution was centrifuged to remove any solid particles. Thereafter, the protein concentration and proteolytic activity were measured. The fraction with good activity and quantity was used in the next step.

2.2.2.2. Ammonium sulphate fractionation (second). The partially purified enzyme (320 ml) from the above step was further purified using ammonium sulphate with saturation ranged from 0% to 80% (5% interval). Then the most active fractions in the ranges of 40–45%, 45–50%, and 50–55% were pooled and combined as one fraction (40–55%) and dialysed overnight at 4 °C against 50 mM sodium acetate buffer with pH 5.0. During dialysis several changes of the dialysis buffer were done. Thereafter, the pooled fraction was used to characterise the enzyme.

2.2.3. Determination of the protein content

The protein content of the fractions obtained after fractionation with ammonium sulphate was estimated by measuring the absorbance at 280 nm. Quantitative determination of the protein content was estimated according to Lowry, Rosebrough, Farr, and Randall (1951) method using BSA as a standard protein.

2.2.4. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done, using the method of Laemmli (1970), with 15% acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. Samples were prepared in a tris–glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 5 h in electrophoretic tris–glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins with 0.2% coomassie brilliant blue-R250. Protein stain was destained with 10% acetic acid containing 20% methanol.

2.2.5. Determination of milk-clotting activity

Milk-clotting activity was determined according to the methods described by Arima, Ya, and Iwasaki (1970) with a slight modification. The substrate (10% skim milk in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.5. The substrate (2.0 ml) was pre-incubated for 5 min at 37 °C, and 0.2 ml of enzyme extract was added, and the curd formation was observed at 37 °C while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit is defined as the amount of enzyme that clots 10 ml of the substrate within 40 min.

$$\text{MCA (U/ml)} = (2400/\text{clotting time (s)}) \times \text{dilution factor}$$

2.2.6. Determination of protease activity

In the preliminary experiment, in order to select an appropriate assay method, three different methods were tried to determine the protease activity of *S. dubium* extract. The colourimetric assay using

azocasein as a substrate described by Sarath, Motte, and Wanger (1989) was the most reliable one and therefore used in this study. About 0.15 ml enzyme solution was added to 0.25 ml of 1% azocasein in 50 mM tris–HCl buffer, pH 8.0. The content of the tubes were mixed gently and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 1.2 ml of 5% TCA, and after incubated at room temperature for 30 min, the tubes were centrifuged at 10,300 rpm for 10 min at 4 °C. One millilitre of the supernatant was mixed with 1.0 ml of 1.0 M NaOH and kept for 10 min at room temperature for colour development. A blank was prepared in the same way, but with the addition of TCA to the enzyme before the substrate. The absorbance was read at 440 nm. One unit of the enzyme activity was defined as the amount of the enzyme produced an increase of the absorbance at 440 nm by 1.0/min under the assay conditions. The proteolytic activity of the extracted enzyme at different concentration using 1% azocasein was carried out as described above. The proteolytic activity of the enzyme at different substrate (azocasein) concentrations was determined. The proteolytic activity against reaction time was also determined as described above.

2.2.7. pH and temperature optima

The activity of partially purified enzyme is measured as a function of varying pH to determine the pH optima of the enzyme. The buffers used are as follows: 50 mM citrate–sodium phosphate (pH 5.0 and 6.0), 50 mM tris–HCl (pH 6.0–8.0), and 50 mM glycine (pH 8.0–12.8 adjusted with NaOH). The substrate solution of azocasein was prepared in the respective buffers and then the pH stability of the enzyme was determined by measuring the remaining activity after incubating the enzyme at 30 °C for 1 and 25 h or at 60 °C for 30 min in the respective buffers. The effect of temperature (20–90 °C) on the activity of the enzyme was also studied using azocasein as a substrate. Prior to the assays, the substrate solution was also equilibrated at the corresponding temperature in 50 mM tris–HCl buffer (pH 8.0). At each temperature, a control assay was carried out without the enzyme and used as a blank. The remaining activity was measured at 30 °C for 30 min and expressed as percentage of the control.

2.2.8. Enzyme activity on synthetic peptides

Protease activity was determined by measuring *p*-nitroaniline liberation from the chromogenic synthetic peptide substrates such as *N*-Suc-Ala-Ala-Ala-*p*NA, *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, and Ac-Tyr-Val-Ala-Asp-*p*NA. Substrates were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 20 mM. The assay was preformed in a total reaction mixture of 1.5 ml. Five different substrates concentrations in 0.2 M tris–HCl, pH 7.5, were pre-incubated at 25 °C for 5 min, and then 16 μg enzyme was added. The reaction was preceded for 5 min and the rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was measured using Shimadzu UV-2100S spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 410 nm. An extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm was used for the yield of the hydrolysis product, *p*-nitroaniline (Erlanger, Kokowsky, & Cohen, 1961). One unit (u) of the enzyme activity was defined as the amount of enzyme that liberated 1 umol of *p*-nitroaniline in 1 min under the assay conditions.

2.2.9. Kinetic constants

Kinetic constants of the purified enzymes were calculated from the product accumulation curves with molar absorption coefficient for *p*-nitroaniline determined in the reaction buffer at 25 °C for different synthetic peptide substrates. At least five different substrate concentrations were used and the steady state kinetic parameters were analysed by employing the Cornish-Bowden (1975) transformation of the Michaelis–Menten equation.

Table 1
Milk-clotting activity of different extractants of *Solanum dubium* seeds enzyme.

Extractant	Milk-clotting activity (U/ml)
Distilled water	180.2
5% NaCl in distilled water	512.8
50 mM acetate buffer (pH 3.8)	575.7
5% NaCl in acetate buffer (pH 5.0)	673.3
5% NaCl in tris–HCl buffer (pH 8.0)	274.8

3. Results and discussion

3.1. Extraction and purification

As shown in Table 1 the extraction of a milk-clotting enzyme from *S. dubium* seeds with 5% NaCl in 50 mM sodium acetate buffer (pH 5.0) gave higher milk-clotting activity compared to that extracted with other buffers. Therefore, this buffer (5% NaCl in sodium acetate buffer, pH 5.0) was used as an extracting buffer throughout the study. Moreover, the enzyme was extracted for a period ranged from 1 to 24 h and it was found that the extraction time had no effect on the enzyme yield and activity (Fig. 2). Both 5% NaCl in 50 mM acetate buffer (pH 5.0) and distilled water were effective in extracting the enzyme from *S. dubium* seeds. However, extraction with 5% NaCl in 50 mM acetate buffer (pH 5.0) yielded higher milk-clotting activity than distilled water. The results obtained agree with Yousif et al. (1996) who observed that the water extract of *S. dubium* berries had lower milk-clotting activity than the 5% NaCl in acetate buffer extract which indicated that an increase in the ionic strength of the extracting solution apparently increases the solubility of the enzyme and enhanced its extraction. Similar results were obtained when a milk-clotting enzyme was extracted from a related plant species, *Solanum torvum*, using 5% NaCl (Hamdy, Sheded, Elkoussy, & Foda, 1976). Yousif et al. (1996) found that the milk-clotting activity was higher in the seed and whole berry extracts than in the berry coat extract, therefore, it could be assumed that the milk-clotting enzyme is located primarily in the fruit sap which surrounds the seeds and when the fruit dries, it will be concentrated in the fruit core around the seeds. The existence of the enzyme in the seeds outer layer makes it easy to be extracted and also decreases the extraction time.

Ammonium sulphate fractionation was performed as a first purification step of the enzyme. The crude extract (510 ml in 50 mM sodium acetate buffer, pH 5.0) was precipitated with ammonium sulphate (0–80% saturation). The results showed that 35–55% saturation gave the highest milk-clotting activity, and

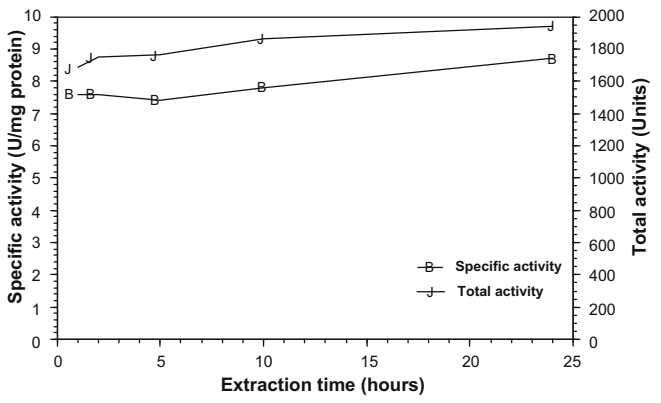

Fig. 2. Effect of extraction time on total and specific activity of partially purified *Solanum dubium* enzyme.

Table 2
Ammonium sulphate fractionation of a milk-clotting protease from *Solanum dubium* seeds (first).

NH_4SO_4	Total activity	Total protein (mg)	Specific activity	Yield (%)	Purification
Crude	65.4×10^3	2.14×10^3	30.6	100.0	1.00
0–35	5.5×10^3	197	28.0	8.4	0.92
35–55	61.0×10^3	968	63.0	93.3	2.06
55–80	2.4×10^3	125	18.8	3.6	0.62

Table 3
Ammonium sulphate fractionation of a milk-clotting protease from *Solanum dubium* seeds (second).

NH_4SO_4 (%)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
35–40	15.4	967.0	63.0	2.3	0.77
40–45	272.2	24.8×10^3	91.0	58.9	1.10
45–50	152.0	14.3×10^3	94.3	34.1	1.15
50–55	45.6	4.4×10^3	95.8	10.4	1.17
55–80	17.5	1.5×10^3	86.8	3.6	1.06
40–55[a]	477.0	40.7×10^3	91.7	63.8	2.90

[a] Combined fraction.

specific activity (Table 2). The results obtained indicated that the degree of saturation of ammonium sulphate greatly affected the enzyme activity, yield and total protein as well as the purification. By applying 35–55% ammonium sulphate fractionation as a first purification step, over 86% of the total protease in the crude extract was salted out. This procedure not only facilitates the effective removal of the brown-coloured materials in the crude extract, but also concentrated the enzyme to a workable volume that could be further subjected to fractionation by ammonium sulphate (35–85% saturated) as a second purification step. To obtain a pure ammonium sulphate fraction with high specific activity, the fractions were collected at 5% saturation intervals over the range of 35–55% saturation. The results obtained showed that ammonium sulphate fractions of 40–45%, 45–50%, and 50–55% saturation had almost the same specific activity (Table 3). Therefore, such fractions were pooled as one fraction (40–55%). The enzyme of the pooled fractions was purified 3.0-folds with a yield of 63.8%, and a specific activity of 91.7 (U/mg protein). The pooled fraction was dialysed overnight against 50 mM sodium acetate buffer (pH 5.0) with several changes of the buffer, and then used for further study. Gel electrophoresis of the extracted enzyme is shown in Fig. 3. It was clear that when the crude extract was subjected to two steps of ammonium sulphate fractionation a partially purified band was obtained of a molecular weight of 66 kDa. It is noteworthy that the recovery obtained during the purification course of the enzyme was high which indicated that the enzyme constituted most of the protein in *S. dubium* seeds.

3.2. Milk-clotting activity

Table 4 summarises the milk-clotting and proteolytic activity of the enzyme compared to other coagulants. As shown, *S. dubium* enzyme had both higher clotting and proteolytic activities compared to other plant enzymes. It was found that the clotting and proteolytic activities of the enzyme were 880 units/ml and 0.35 (OD 660 nm), respectively, and that of rennet were 249.6 units/ml and 0.05 (OD 660 nm), respectively. The results obtained indicated that *S. dubium* enzyme was highly active compared to rennet. As shown in Fig. 4 the partially purified enzyme greatly coagulated the skim milk compared to crude extract and the control sample.

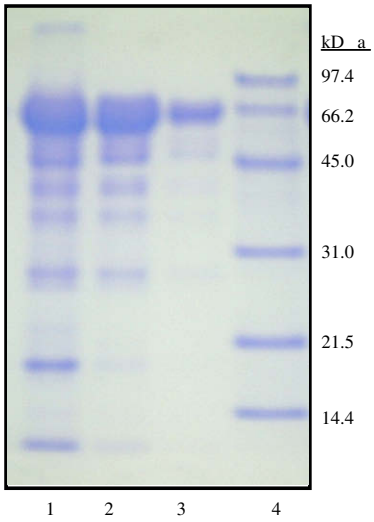

Fig. 3. SDS–PAGE pattern of partially purified enzyme from *Solanum dubium* seeds. Lane 1, crude extract; lane 2, first ammonium sulphate; lane 3, second ammonium sulphate; and lane 4, molecular markers.

Table 4
Ratio of milk-clotting activity/proteolytic activity of *Solanum dubium* seeds enzyme and other coagulants.

Enzyme	Clotting activity (units/ml)	Proteolytic activity (OD 660 nm)	Ratio (units/OD 660 nm)
Rennet	249.6	0.05	4992
Mucor rennet	551	0.11	4650
Endothia parstica enzyme	750	0.29	2590
Solanum dubium enzyme	880	0.35	2490
Papain	216	0.59	367

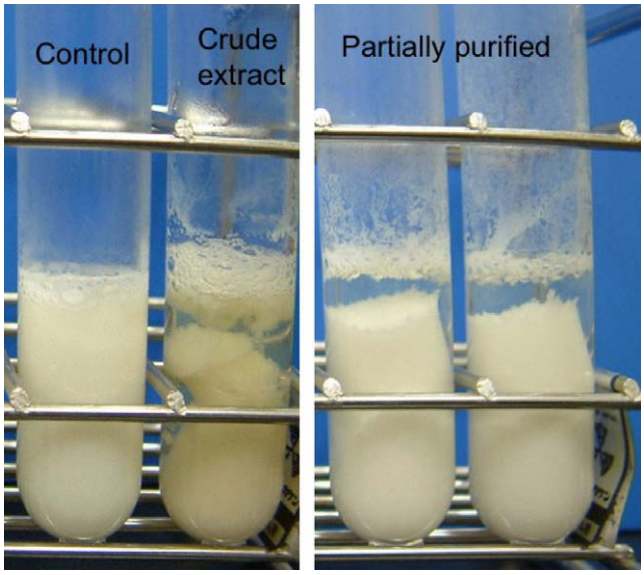

Fig. 4. Coagulation of skimmed milk by crude and partially purified enzyme from *Solanum dubium* seeds.

3.3. Proteolytic activity against enzyme or substrate concentration

The proteolytic activity of *S. dubium* enzyme was highly increased with increase in enzyme concentration (Fig. 5) and also

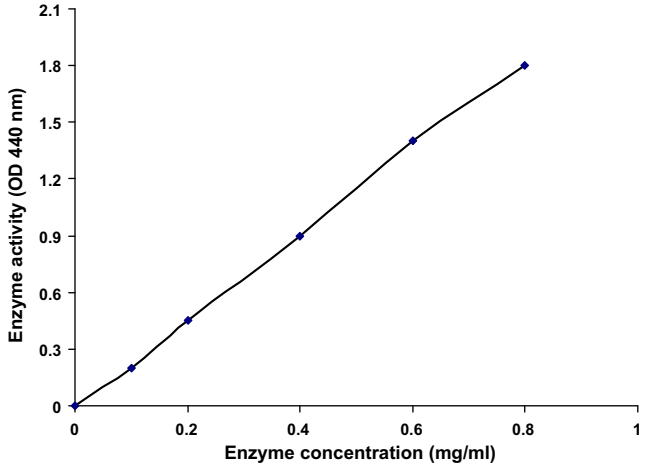

Fig. 5. Activity of partially purified enzyme of different concentration.

with increase in substrate concentration (Fig. 6). Moreover, a positive and linear correlation was observed between the proteolytic activity and the enzyme concentration. The results obtained indicated that the extracted enzyme was very active even for a long time. The results obtained are comparable to those of cucumisin-like serine proteases from *Cucumis trigonus* Roxburghi (Asif-Ullah, Kim, & Yu, 2006), *Cucumis melo* L. var. Prince (Yamagata, Ueno, & Iwasaki, 1989), *Euphorbia milii* (Yadav & Jagannadhan, 2006), and *Trichosantus kirrilowi* (Uchikoba, Horita, & Kaneda, 1990).

3.4. Substrate specificity of the enzyme

In order to determine the substrate specificity of the purified protease, its ability to hydrolyse a number of synthetic substrates were tested. The kinetic parameters were determined and are reported in Table 5. The protease efficiently hydrolysed the substrates *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, and Ac-Tyr-Val-Ala-Asp-*p*NA, which are readily hydrolysed by subtilisins and chymotrypsin-like serine proteases (Fontanini & Jones, 2002). The best substrate for the purified enzyme was Ac-Tyr-Val-Ala-Asp-*p*NA, which is the most favoured substrate for cucumisin (Arima, Yonezawa, Uchikoba, Shimada, & Kaneda, 2000). Whereas, *N*-Suc-Ala-Ala-Ala-*p*NA is the specific substrate for elastase and was not hydrolysed by this protease. The results indicated that the purified protease preferred both hydrophilic and hydrophobic amino acid residues at the P1 position. Thus, the specificity of *S.*

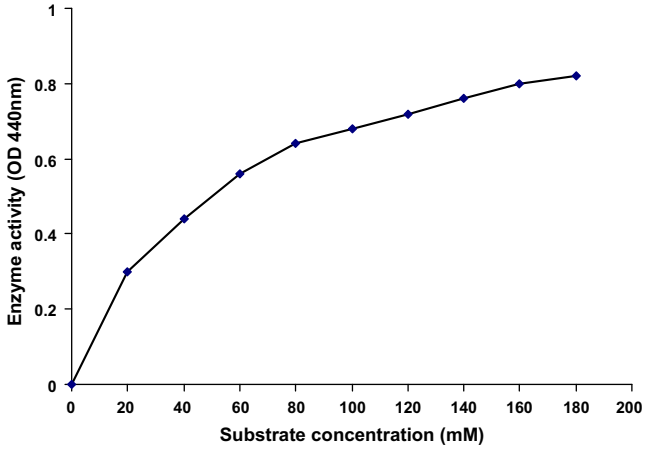

Fig. 6. Effect of substrate concentration on *Solanum dubium* seeds enzyme activity.

Table 5
Substrate specificity of *Solanum dubium* enzyme on different peptide substrates.

Substrate	Substrate concentration (mM)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
N-Suc-Ala-Ala-Pro-Phe-*p*NA	2.0–0.25	1.54	6.9×10	$4.90 \times 10^{+4}$
N-Suc-Ala-Ala-Pro-Leu-*p*NA	2.0–0.25	5.00	$1.9 \times 10^{+2}$	$3.97 \times 10^{+4}$
Ac-Tyr-Val-Ala-Asp-*p*NA	1.0–0.25	1.39	$4.3 \times 10^{+2}$	$3.09 \times 10^{+5}$
N-Suc-Ala-Ala-Ala-*p*NA	2.0–0.25	0	0	0

dubium serine protease is differs from that of cucumisin, a well-known and characterised serine protease from the latex of *C. melo* (Arima et al., 2000). The preference of hydrophobic residue at the P1 position of this enzyme was comparable to that of chymotrypsin, and subtilisin (Hedstrom, 2002). The catalytic efficiency of the purified protease was enhanced by an aliphatic amino acids (Leu) compared to an aromatic residue (Phe) at the P1 position at the same site. Moreover, aliphatic neutral residues (e.g., Ala, Pro) are preferred at the P2 site as seen also for the other proteases (Yamagata et al., 1989). The K_m values for the enzyme were estimated to be 1.39, 1.54, and 5.0 mM against Ac-Tyr-Val-Ala-Asp-*p*NA, *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, respectively. The K_m value of the enzyme with Asp at P1 position was slightly lower than with Phe and both were three times lower than with Leu. These results indicated that the enzyme preferred a non-charged residue at the P1 position to a non-polar residue at the same position. Generally, the specificity of serine proteases is judged by at least five residues (P1–P5) of a substrate. From these residues, P1, P2, and P5 were found to have much impact on the enzyme hydrolytic activity of the substrate (Hedstrom, 2002). In this study, the enzyme showed a preference for a non-charged group rather than a negatively charged one at the P5 position, since the substrate with Ac-group at P5 had higher K_{cat}/Km. In contrast to our results, catepsin G was found to prefer substrates with a negatively charged residue rather than a neutral one at the P5 position. In another study, besides its preference to both hydrophobic and hydrophilic residues at the P1 position, the enzyme showed broad substrate specificity with different casein fractions (data not shown). One of the notable aspects of serine proteases is their wide diversity of substrate specificities coupled to a single catalytic mechanism.

3.5. Effect of pH on partially purified enzyme activity

As shown in Fig. 7, the purified enzyme is stable under a wide range of pH and it retained all of its activity in the pH range from

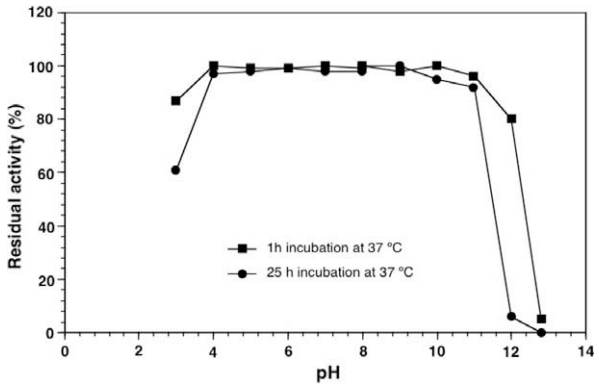

Fig. 7. Effect of pH on the stability of partially purified enzyme from *Solanum dubium* seeds.

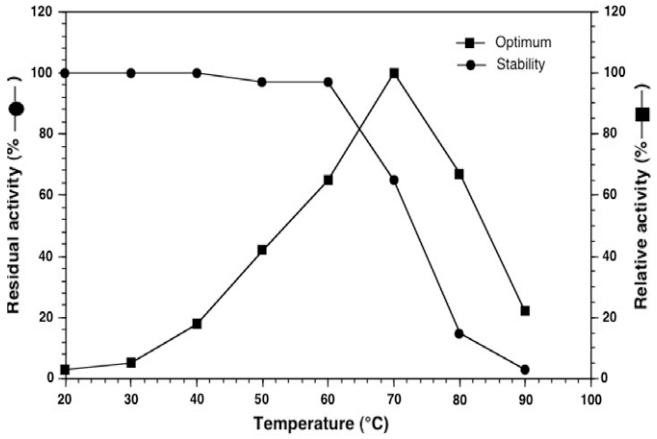

Fig. 8. Effect of temperature on the activity and stability of partially purified enzyme from *Solanum dubium* seeds.

4.0 to 11.0 when it was incubated for 1 or 25 h at 37 °C. However, when it was incubated at a pH range of 3.0–12.0 at 30 °C for 1 h more than 80% of the activity retained. The results obtained showed that the isolated enzyme is more stable at a wide range of pH (3.0–12.0), and its stability is more comparable to those of cucumisin-like serine proteases from *C. trigonus* Roxburghi (Asif-Ullah et al., 2006), *C. melo* L. var. Prince (Yamagata et al., 1989), *E. milii* (Yadav & Jagannadhan, 2006), and *T. kirrilowi* (Uchikoba et al., 1990). In this regards, the isolated enzyme is unique, and therefore might be suitable for application in the food industry under alkaline conditions. The stability of the enzyme against pH is an important characteristic because most of plant enzymes are catalytically unstable at alkaline pH values, thus limiting their usefulness in food industry especially as cheese making coagulants (Lamas, Barros, Balcao, & Malcata, 2001).

3.6. Effect of Temperature on partially purified enzyme activity

The results obtained in Fig. 8 showed that the enzyme activity increased as the temperature increased from 20 to 70 °C. The activity at 70 °C was 5- and 10-fold higher than that of the activity at 40 and 20 °C, respectively. The activity rapidly decreased as the temperature raised over 80 °C. Since the enzyme had a high optimum temperature, its stability at a temperature ranged from 20 to 90 °C was studied. There was a total retention of activity after 1 h incubation at 60 °C and about 70% of its activity was retained at 70 °C when the enzyme was incubated for 1 h. The thermostability of the enzyme was found to be up to 70 °C. The temperature profile of the enzyme was agreed with those of subtilisin/cucumisin like plant serine proteases reported by Asif-Ullah et al. (2006), Yamagata et al. (1989), and Uchikoba et al. (1990).

In conclusion, compared to other purification procedures done we concluded that a simple purification procedure has been developed in this study to obtain a very active and stable enzyme from *S. dubium* seeds for milk-clotting. However, further purification is required to get a highly pure enzyme.

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